REMARKS

Entry of the foregoing, reexamination and further and favorable reconsideration of the subject application in light of the following remarks are respectfully requested.

By the foregoing amendment, Claims 1, 2, 4, 5, 7, 14, 17, 20-21 and 23 have been amended to place the subject application in a better condition for allowance. Support for the amendments can be found throughout the specification. Claim 22 has been canceled without prejudice to or disclaimer of the subject matter recited therein. No new matter has been added.

Substitute Specification:

Turning now to the Official Action, the Examiner has objected to the substitute specification filed 19 February, 2002 for allegedly not containing a marked-up copy showing the amendments made. Applicants hereby submit another substitute specification in proper idiomatic English and in compliance with 37 C.F.R. § 1.52(a) and (b) along with this response. A marked-up copy of the specification, showing the amendments made via the substitute specification, is attached. For clarity and completion, Applicants have also included amendments to the specification from the Amendment and Reply filed on February 6, 2001, already made of record by the Examiner. In particular, a paper copy of the sequence listing (filed on February 6, 2001) has been added after the last page of the substitute specification, currently page 27. Please amend the remaining page numbers accordingly. This substitute specification is in full compliance with the sequence listing rules set forth in 37 C.F.R. §§ 1.821-.825. Applicants submit that no new matter has been

added with regard to the substitute specification. Entry of this substitute specification is respectfully requested.

Claims Objections:

The Examiner has objected to Claims 1, 4, 14, 17, 20-21 and 27 as purportedly containing informalities. In order to expedite prosecution in the subject application, and not to acquiesce to the Examiner's objection, Applicants have amended these claims to comply with the Examiner's suggestions. Withdrawal of this objection is respectfully requested.

Claims Rejected Under 35 U.S.C. § 112, First Paragraph:

The Examiner has rejected Claims 1-2, 4-5, 7, 13-14, 16-17, 20-23 and 27-28 under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for nucleic acids of SEQ ID NO:2 or those encoding SEQ ID NO:1 and plant cells and plants transformed with those nucleic acids, allegedly does not reasonably provide enablement for nucleic acids that encode SEQ ID NO:1, encode modified nucleic acids or that hybridize under unspecified stringency to nucleic acids that encode SEQ ID NO:1.

Specifically, the Examiner alleges that undue experimentation is needed because the specification fails to provide guidance regarding which amino acids can be deleted or which regions of the protein can tolerate insertions to continue producing a functional enzyme. Applicants respectfully traverse this rejection.

MPEP § 2164 recites that the disclosure must "contain sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention. . ." without undue experimentation.

It is well within the purview of the skilled artisan and the teachings of the subject specification to modify nucleotide sequences by deletion, substitution or insertion and then determine if such modified sequences maintain the desired enzymatic activity. Further, it is well within the purview of the skilled artisan to produce modified nucleotide sequences and determine if such modified sequences hybridize to the sequence of SEQ ID NO:1 under stringent conditions. Furthermore, it is well within the purview of the skilled artisan to determine if proteins encoded by these modified nucleic acids maintain the recited enzymatic activities. Applicants further submit that the specification, at pages 35-39 (Examples 9-10), teaches how to measure activity of encoded protein. These types of experimentation are merely routine and do not constitute undue experimentation. The specification need not teach what is known in the art (e.g., modifying nucleic acid sequences). In fact, the Federal Circuit has stated that a patent need not teach, and preferably omits, what is well known in the art. *See Hybritech, Inc. v. Monoclonal Antibodies, Ind.*, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986).

However, in order to expedite prosecution in the subject application, and not to acquiesce to the Examiner's rejection, Applicants have amended Claims 1 and 4, part (b), to recite "a modified nucleotide sequence which hybridizes under stringent conditions to the complementary sequence of said nucleotide sequence (a). . . ." The specification, at pages 7-11, clearly discloses the method for making a modified SEQ ID NO:1, using

hybridization under stringent conditions, encoding a polypeptide which maintains all of the N-methyl transferase enzyme activities. Therefore, modification of such amino acid does not involve undue experimentation.

Additionally, the Examiner alleges that SEQ ID NO:1 does not appear to be the entire protein sequence. The Examiner alleges that "there is no evidence to suggest that a nucleic acid encoding only SEQ ID NO:1 would function to encode an enzyme with the listed properties, especially since the starting ATG is missing." Applicants respectfully traverse.

Applicants direct the Examiner's attention to the specification, Example 9 - expression of N-methyl transferase in *E. Coli*; (page no. 35). The results shown by this example clearly provide that although SEQ ID NO:1 has not the entire sequence, SEQ ID NO:1 functions to encode an enzyme with the listed properties, even with the starting ATG sequence missing. The specification at page 37, lines 16-19, states that by using the procedures disclosed in Example 9 the encoded isolated enzyme (SEQ ID NO:1), had the listed properties of three different N-methyl transferase activities. Thus in light of the foregoing withdrawal of this rejection is respectfully requested.

The Examiner has rejected Claims 20-23 under 35 U.S.C. § 112, first paragraph, as purportedly lacking enablement. The Examiner alleges that anti-sense suppression of genes is very unpredictable, therefore, it is not certain that such gene will inhibit sense gene transcription or secondary metabolite levels when transformed into a plant of a different species. Furthermore, the Examiner alleges that the specification does not teach

production of "ANY" plant metabolite, nor does it provide guidance for altering the "composition" of any plant metabolite.

"As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. § 112 is satisfied." *In re Fischer*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970).

"During examination, the claims must be interpreted as broadly as their terms reasonably allow. This means that the words of the claim must be given their plain meaning unless applicant has provided a clear definition in the specification." *In re Zletz*, 893 F.2d 319, 321, 13 USPQ2d 1320, 1322 (Fed. Cir. 1989).

Applicants submit that it is well within the purview of the skilled artisan and the teachings of the subject specification to make and use anti-sense suppression genes that are not completely homologous to the target gene which will inhibit sense gene transcription or secondary metabolite levels when transformed into a plant of a different species. Applicants direct the Examiner's attention to the specification, at page 25, regarding making and using sense or anti-sense DNA to obtain transformed plants for purpose of improving productivity for specific compounds by modifying the metabolism of such compound in the host plant.

Furthermore, Applicants direct the Examiner's attention to the specification, Example 10 - suppression of caffeine synthesis according to and anti-sense method; (page no.37). This Example illustrates the make and use of a recombinant vector carrying an ani-sense N-methyl transferase gene which resulted in a significant reduction in production

of the plant secondary metabolite, caffeine, in a caffeine producing plant. The specification discloses one method for making and using the claimed invention that bears reasonable correlation to the entire scope of the claim. Therefore, the enablement requirement of 35 U.S.C. § 112 is satisfied.

Applicants further disagree with the Examiner that the instant specification relates to "ANY" plant metabolite. Applicants direct the Examiner's attention to the specification at page 25, second paragraph, for the definition of secondary metabolites. To prevent uncertainty or ambiguity Applicants, in the specification, have disclosed the compounds that a plant secondary metabolites encompass by using Markush Groups. It is clearly stated that "a plant secondary metabolite is selected from a group consisting of 7-methyl xanthine, paraxanthine, theobromine, and caffeine." This means that Applicants have provided a clear definition of the term "a plant secondary metabolite" in the specification.

However, to expedite prosecution in the subject application and not to acquiesce to the Examiner's rejection, Applicants have amended Claims 20, 21 and 23 to further clarify the claimed invention. Claim 20 has been amended to incorporate the limitation of Claim 22. Therefore, it limits the phrase "a secondary plant metabolite" to consist of 7-methyl xanthine, paraxanthine, theobromine, and caffeine. Claim 21 has been amended to limit the phrase "a secondary plant metabolite" to only caffeine. Also, the phrase "modifying a composition" has been replaced by "modifying a concentration" as recommended by the examiner. The phrase "modifying the concentration of caffeine" derives support, at least, from the specification, Example 10; (page no. 37).

In light of the forgoing amendments and arguments, Applicants respectfully request the withdrawal of this rejection.

The Examiner has rejected Claims 1-2, 4-5, 7, 13-14, 16-17, 20-23 and 27-28 under 35 U.S.C. § 112, first paragraph, as purportedly lacking a written description. The Examiner alleges that the specification "fails to describe the structural features of these modified nucleic acids." Specifically, the Examiner has stated that the claims are broadly drawn to a multitude of DNA molecules, however, the specification only describes a coding sequence from *Camellia sinensis* that comprises SEQ ID NO:2, which encodes a full-length enzyme. Based thereon, the Examiner has stated that Applicant has not described DNA molecules that encode an N-methyl transferase within the full scope of the claims, and the specification fails to provide an adequate written description of the claimed invention. The Examiner further alleges that "one skilled in the art would not have been in possession of the *genus* claimed at the time this application was filed." (Emphasis added)

> "[I]f an applicant disclosed an amino acid sequence, it would be unnecessary to provide an explicit disclosure of nucleic acid sequences that encoded that amino acid sequence. Since the genetic code is widely known, a disclosure of an amino acid sequence would provide sufficient information such that one would accept that an applicant was in possession of the full *genus* of nucleic acids encoding a given amino acid sequence..." (Emphasis added)

In re Bell, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993)

Applicants direct the Examiner's attention to the specification at pages 10-15 regarding the structural features and stringent hybridization conditions relating to these modified nucleic acids. Applicants submit that it is well within the purview of the skilled artisan to modify nucleotide sequences by deletion, substitution or insertion and then

determine if such modified sequences maintain the desired enzymatic activity. Further, it is well within the purview of the skilled artisan to produce modified nucleotide sequences and determine if such modified sequences hybridize to the sequence of SEQ ID NO:1 under stringent conditions. Furthermore, it is well within the purview of the skilled artisan to determine if proteins encoded by these modified nucleic acids maintain the recited enzymatic activities. The genetic code is widely known and these types of experimentation are merely routine and do not constitute undue experimentation. The specification need not teach what is known in the art (e.g., modifying nucleic acid sequences). In fact, the Federal Circuit has stated that a patent need not teach, and preferably omits, what is well known in the art. *See Hybritech, Inc. v. Monoclonal Antibodies, Ind.*, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986).

Therefore, Applicants respectfully request withdrawal of the rejection of claims 1-2, 4-5, 7, 11-14 and 16-23 under 35 U.S.C. § 112, first paragraph.

Claims Rejected Under U.S.C. § 112, Second Paragraph:

The Examiner has rejected Claims 1-7, 11-14, 16-23 and 27-28 under U.S.C. § 112, second paragraph, as purportedly indefinite.

Claims 1 and 4 are rejected "because it is not clear if the DNA or RNA comprises both or either parts (a) and (b)." In order to expedite prosecution in the subject application, and not to acquiesce to the Examiner's rejection, Applicants have amended Claims 1 and 4 by replacing the phrase "any of the following" by phrase "either of the following."

Claims 1(b) and 4(b) remain rejected for reciting "obtained by carrying out nucleotide replacement, deletion, or insertion." The Examiner alleges these claims are indefinite because they do not adequately limit the number of nucleotide which are replaced or inserted.

Applicants assert that in light of the amendments made to Claims 1 and 4, and without conceding to the Examiner's rejection, the phrase "obtained by carrying out nucleotide replacement, deletion, or insertion" has been deleted without prejudice to or disclaimer of the subject matter therein. This rejection is thus respectfully moot.

Claims 1(b) and 4(b) are rejected for reciting "said modified sequence." The Examiner alleges that there are no antecedent basis for this phrase. Applicants have amended Claims 1(b) and 4(b) to recite "said modified nucleotide sequence" instead of "said modified sequence."

Claims 1(b) and 4(b) are rejected for reciting "said enzyme activities." The Examiner purports that there are no antecedent basis for this phrase. In order to expedite prosecution in the subject application, and not to acquiesce to the Examiner's rejection, Applicants have amended Claims 1(b) and 4(b) to recite "said N-methyl transferase enzyme activities" instead of "said enzyme activities."

Claims 1(b) and 4(b) are further rejected for reciting "maintains said enzyme activities." The Examiner is allegedly not clear on what maintenance of an enzyme activity means. In order to expedite prosecution in the subject application, and not to acquiesce to the Examiner's rejection, Applicants have amended Claims 1(b) and 4(b) to read "maintains all of said N-methyl transferase enzyme activities" instead of "maintains said

enzyme activities." Claims 2 and 5 have been rejected for reciting "hybridized at a ... to overnight." The Examiner alleges that the claims are not clear as to the level of stringency required. In order to expedite prosecution in the subject application, and not to acquiesce to the Examiner's rejection, Applicants have amended these claims to recite "hybridized under stringent conditions at."

Claims 2 and 5 are further rejected for reciting "said nucleotide sequence (a) and said nucleotide sequence (b)." The Examiner alleges that this particular wording makes it appear that parent claim have both sequence (a) and (b). Furthermore, the Examiner alleges that there is no antecedent basis for these claims as presently recited. In order to expedite prosecution in the subject application, and not to acquiesce to the Examiner's rejection, Applicants have amended Claims 2 an 5 to recite "in claim 4 wherein" instead of "in claim 4, wherein."

Claim 7 is rejected for reciting "said N-methyl transferase encoded by the DNA molecule." The Examiner alleges that there is no antecedent basis for Claim 7 as presently recited. In order to expedite prosecution in the subject application, and not to acquiesce to the Examiner's rejection, Applicants have amended Claim 7 to recite "N-methyl transferase encoded by the DNA molecule" instead of "said N-methyl transferase encoded by the DNA molecule."

Claim 23 is rejected for reciting "a transformed whole plant" allegedly without antecedent basis. The Examiner purports that the parent claim 20 makes no reference to a transformed whole plant. In order to expedite prosecution in the subject application, and not to acquiesce to the Examiner's rejection, Applicants have amended both Claim 20 and

23. Claim 20 has been amended to include the phrase "or whole plant." Claim 23 has been amended to read "said transformed whole plant" instead of "a transformed whole plant."

Withdrawal of this rejection is thus respectfully requested.

From the foregoing, further and favorable reconsideration in the form of a Notice of Allowance is believed to be next in order and such action is earnestly solicited.

In the event that there are any questions concerning this amendment, or the application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of this application may be expedited.

Respectfully submitted,

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Date: September 3, 2002

Attachment to Reply and Amendment dated September 3, 2002

Marked-up Claims 1, 2, 4, 5, 7, 14, 17, 20, 21, 23, 27

1. (Twice Amended) An isolated DNA molecule comprising either ~~any~~ of the following nucleotide sequences:

(a) a nucleotide sequence encoding N-methyl transferase of SEQ ID NO:1 and having the N-methyl transferase ~~N-methyltransferase~~ enzyme activities of 7-methylxanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase; or

(b) a modified nucleotide sequence ~~obtained by carrying out nucleotide replacement, deletion, or insertion in~~ which hybridizes under strtingent conditions to the complementary sequence of said nucleotide sequence (a) where the ~~a~~ polypeptide encoded by said modified nucleic sequence maintains all of said N-methyl transferase enzyme activities.

2. (Twice Amended) The isolated DNA molecule as claimed in claim 1~~,~~ wherein said nucleotide sequence (a) and said modified nucleotide sequence (b) can be hybridized under stringent conditions at a temperature ranging from 40° to 80°C for a time period ranging from several hours to overnight.

4. (Twice Amended) An isolated RNA molecule comprising either ~~any~~ of the following nucleotide sequences:

Attachment to Reply and Amendment dated September 3, 2002

Marked-up Claims 1, 2, 4, 5, 7, 14, 17, 20, 21, 23, 27

(a) a nucleotide sequence encoding N-methyl transferase of SEQ ID NO:1 and having the N-methyl transferase ~~N-methyltransferase~~ enzyme activities of 7-methylxanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase; or

(b) a modified nucleotide sequence ~~obtained by carrying out nucleotide replacement, deletion, or insertion in~~ which hybridizes under stringent conditions to the complementary sequence of said nucleotide sequence (a) where the ~~a~~ polypeptide encoded by said modified nucleic sequence maintains all of said N-methyl transferase enzyme activities.

5. (Twice Amended) The isolated RNA molecule as claimed in claim 4~~,~~ wherein said nucleotide sequence (a) and said modified nucleotide sequence (b) can be hybridized under stringent conditions at a temperature ranging from 40° to 80°C for a time period ranging from several hours to overnight.

7. (Thrice Amended) An expression vector comprising the DNA molecule as claimed in claim 1 and a promoter for expressing ~~said~~ N-methyl transferase encoded by the DNA molecule in plant cells.

Attachment to Reply and Amendment dated September 3, 2002

Marked-up Claims 1, 2, 4, 5, 7, 14, 17, 20, 21, 23, 27

14. (Twice Amended) The vector as claimed in claim 13, wherein the vector ~~encodes~~ expresses an N-methyl transferase with 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase, and paraxanthine N3 methyl transferase activities in cells of at least one of microorganisms or plants.

17. (Thrice Amended) The ~~A~~ plant cell, plant tissue, or whole plant as claimed in claim 16, wherein the vector is introduced by infection.

20. (Twice Amended) A method for producing a plant secondary metabolite selected from the group consisting of 7-methyl xanthine, paraxanthine, theobromine and caffeine comprising: culturing the transformed plant cell, ~~or~~ plant tissue or whole plant as claimed in claim 16 to form a plant body, and culturing said plant body to produce a plant secondary metabolite, wherein said plant cell, ~~or~~ plant tissue or whole plant is a Camellia or a Coffea ~~coffea~~ plant cell, ~~or~~ plant tissue or whole plant.

21. (Thrice Amended) A method for modifying ~~a composition~~ the concentration of ~~a plant secondary metabolite~~ caffeine comprising: culturing the plant cell or plant tissue as claimed in claim 16 to form a plant body, and culturing said plant body to modify a composition of ~~a plant secondary metabolite~~ caffeine, wherein said plant cell or plant tissue is a Camellia or a ~~coffea~~ Coffea plant cell or plant tissue.

~~22. (Twice Amended) The method as claimed in claim 20, wherein the plant secondary metabolite is at least one or more compounds selected from the group consisting of 7-methyl xanthine, paraxanthine, theobromine, and caffeine.~~

23. (Thrice Amended) The ~~A~~ method as claimed in claim 20, wherein said ~~a~~ transformed whole plant is a Camellia plant or a Coffea plant.

27. (Amended) A vector encoding the ~~an~~ RNA molecule as claimed in claim 4.

SPECIFICATION

Title of the Invention

Gene Encoding Caffeine Synthesis System Associated Enzyme and Use Thereof

1. Field of the Invention

The present invention relates to a N-methyl transferase, one of the enzymes constituting a caffeine metabolic system, which is a polypeptide simultaneously having activities of three methyl transferases, 7-methyl xanthine N3 methyl transferase, theobromine Nl methyl transferase and paraxanthine N3 methyl transferase, and variants of the N-methyl transferase; DNA molecules or RNA molecules having nucleotide sequences encoding any one of the N-methyl transferase and the variants thereof; vectors using these molecules; and cells transformed with the vectors and their uses.

2. Description of the Related Art

Caffeine is a purine alkaloid contained in Theaceae Camellia plants such as Camellia sinensis or Rubiaceae Coffea plants such as Coffea arabica or the like, and is used as a raw material for medicines or a food additive. At present, caffeine is produced by extraction from caffeine [productive] producing plants including the foregoing plant species or organic synthesis. In addition, in [gusts] items such as tea or coffee, in order to alleviate or enhance their stimulus, a reduction or increase in the content of caffeine and its intermediates is attempted using classical bleeding techniques or the like.

In Phytochemistry, 31, 2575- (1992), there is disclosed [experiments using ^{14}C-tracer] a reaction pathway indicating that caffeine is iosynthesized from xanthosine through three stages of N-methylation. [This reaction path is shown below] This was determined by conducting experiments using a ^{14}C-tracer.

Enzyme activities for catalyzing this methylation, that is, methyl transferase activities, were first reported by a study using coarse extraction of tea leaves in 1975 (Biochem. J. 146, 87- (1975)). For tea, partial purification of methyl transferase was reported (Physiol. Plant., 98, 629-(1996)), but no enzyme protein was isolated. Although attempts have been made to purify methyl transferase from coffee (Phytochemistry, 37, 1577- (1994)), the purification magnification is very low.

As described above, concerning the amino acid sequence of the enzyme in a caffeine metabolic system, that is, a N-methyl transferase that catalyzes a two- stage methylation reaction from 7-methyl xanthine through theobromine to caffeine that is a final reaction of caffeine biosynthesis, neither amino acid sequence nor DNA encoding the amino acid sequence is known in the prior art.

Summary of the Invention

It is an object of the present invention to provide N-methyl transferase, one of the enzymes that constitute a caffeine metabolic system useful for caffeine synthesis, simultaneously having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine Nl methyl transferase, and paraxanthine N3 methyl transferase; a DNA or RNA molecule encoding the N-methyl transferase useful for enhancement or suppression of caffeine production in microorganisms or plants, and vectors or the like using the same.

For example, all or part of the DNA molecule according to the present invention is incorporated in microorganisms or plant cells in a form of sense or antisense, thereby making it possible to achieve the following objects:

(1) Efficiently producing N-methyl transferase that can be utilized as an enzyme for industrial, food, or medical use;

(2) Efficiently producing compounds related to caffeine metabolism by modifying caffeine biosynthesis and metabolism of caffeine [productive] producing plants, plant tissues or plant cells; and

(3) Modifying caffeine biosynthesis and metabolism of caffeine productive plants, plant tissues, or plant cells, thereby modifying the production rate of the caffeine metabolism related compounds.

The present inventors conducted N-terminal amino acid sequence analysis of a N-methyl transferase, as a polypeptide simultaneously having activities of three enzymes, 7-methyl xanthine N3 methyl transferase, theobromine Nl methyl transferase and paraxanthine N3 methyl transferase, from cotyledons of tea. Based on the result, a DNA probe was prepared, and a target DNA molecule [were] was successfully isolated by RT-PCR techniques and 5' RACE techniques using this probe.

PCR primers were designed based on the sequence of the N-methyl transferase DNA molecule from tea and used for RT-PCR and 5'-RACE to isolate a DNA from tea cotyledons, which encoded a N-methyl transferase as a peptide which simultaneously has activities of three enzymes, 7-methyl xanthine N3 methyl transferase, theobromine Nl methyl transferase and paraxanthine N3 methyl transferase.

Next, the DNA molecule was integrated into a vector and inserted into Escherichia coli to express a large amount of a polypeptide derived from the DNA molecule. When the expressed polypeptide was recovered and its enzymological properties were investigated, the same reaction as that of the polypeptide having activities of the above described three N-methyl transferases isolated from cotyledons of tea, that is, caffeine production from paraxanthine was observed. It was thus verified that the DNA molecule has a gene that encodes a N-methyl transferase, one of the enzymes [constituting a] of the caffeine synthesis system, simultaneously having activities of the above described three N-methyl transferase.

In plants in which N-methylation is carried out for a xanthosine or analogous compound, or a xanthine or analogous compound, using S-adenosyl methionine (SAM) as a methyl-group donor, polypeptides having the activity of the N-methyl transferase according to the present invention or the same enzyme activity as the transferase and DNAs that encode these peptides are estimated to be contained. Using the method according to the present invention, the N-methyl transferase or

the substantially same enzyme as the transferase and DNA or RNA molecules that encode these transferases can be obtained from these plants.

The present inventors achieved the present invention based on the above findings. That is, the present invention includes the following aspects.

The first DNA molecule according to the present invention are characterized by comprising any one of the following nucleotide sequences:

(a) a nucleotide sequence that encodes a N-methyl transferase, as a polypeptide having an amino acid sequence of SEQ ID NO: 1 of the sequence listing and having enzyme activities of 7 methyl xanthine N3 methyl transferase, theobromine Nl methyl transferase and paraxanthine N3 methyl transferase; and

(b) a modified nucleotide sequence obtained by nucleotide replacement, deletion or insertion in the above described nucleotide sequence (a) within a range where a polypeptide encoded by the nucleotide sequence (a) can maintain the enzyme activity.

It is preferable that the modified nucleotide sequence (b) can be hybridized with the complementary sequence of the nucleotide sequence (a) under stringent conditions.

The first RNA molecule according to the present invention are characterized by comprising any one of the following nucleotide sequences:

(a) a nucleotide sequence that encodes a N-methyl transferase, as a polypeptide having an amino acid sequence of SEQ ID NO: 1 of the sequence listing and having enzyme activities of 7 methyl xanthine N3 methyl transferase, theobromine Nl methyl transferase and paraxanthine N3 methyl transferase; and

(b) a modified nucleotide sequence obtained by nucleotide replacement, deletion or insertion in the above described nucleotide sequence (a) within a range where a polypeptide encoded by the nucleotide sequence (a) can maintain the enzyme activity.

It is preferable that the modified nucleotide sequence (b) can be hybridized with the complementary sequence of the nucleotide sequence (a) under stringent conditions.

A vector for expression of the N-methyl transferase according to the present invention is characterized by comprising the above described first DNA or RNA molecule and a constitution to express the N-methyl transferase encoded by the DNA or RNA molecule, in plant cells. Host cells can be transformed using the expression vector to obtain transformed cells. Further, the transformed cells are cultured, thereby making it possible to produce N-methyl transferase having the above enzyme activities.

Another aspect of a DNA molecule according to the present invention is the second DNA molecule having a sequence complementary to all or a part of the nucleotide sequence of the above first DNA molecule, characterized by that the second DNA molecule is capable of inhibiting the enzyme activities of the plant cells when it is introduced into plant cells having the enzyme activities and expressed in the plant cells.

Another aspect of a RNA molecule according to the present invention is the second RNA molecule having a sequence complementary to all or a part of the nucleotide sequence of the above first RNA molecule, characterized by that the second RNA molecule is capable of inhibiting expression of the N-methyl transferase activities of the plant cells when it is introduced into plant cells having the N-methyl transferase activities and its expression is conducted.

An aspect of a vector according to the present invention is characterized by comprising one of the above DNA molecules and RNA molecules. This vector can be provided so as to have a function either to make expression of the N-methyl transferase possible or to inhibit expression of the N-methyl transferase in a microorganism or a plant , which transferase has the three activities of 7-methyl xanthine N3 methyl transferase, theobromine Nl methyl transferase and paraxanthine N3 methyl transferase as described above.

Using the vector, microorganisms, plant cells, plant tissues, or plant bodies can be transformed, and the obtained transformants are included in the present invention. A secondary metabolite in plants can be produced using the transformed plant cells, plant tissues, or plant bodies. In addition, the composition of the

secondary metabolite in the transformed plants can be modified using the plant cells, plant tissues, or plant bodies.

The N-methyl transferase according to the present invention is a polypeptide having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase, characterized by having:

(a) an amino sequence of SEQ ID NO: 1 of the sequence listing; or

(b) a modified amino acid sequence obtained by replacement, insertion, or deletion of amino acids for the amino acid sequence of SEQ ID NO: 1 of the sequence listing within a range where the above described enzyme activities are not lost, but maintained.

As this modified amino acid sequence (b), it is preferable that the DNA encoding the amino acid sequence (a) and the DNA encoding this modified amino acid sequence (b) can be hybridized under stringent conditions.

According to the present invention, there is provided a DNA molecule and a RNA molecule encoding the N-methyl transferase, which is:

i) one of the enzymes constituting a caffeine synthesis system,

ii) useful for caffeine synthesis and modification of the composition of caffeine produced in microorganisms or plants, and

iii) a polypeptide simultaneously having the three enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase, and paraxanthine N3 methyl transferase.

Detailed Description of the Invention and Preferred Embodiments

The N-methyl transferase according to the present invention is a polypeptide simultaneously having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase.

As for the N-methyl transferase, that [having] has an amino acid sequence indicated by SEQ ID NO: 1 and [that] having a modified amino acid sequence obtained by carrying out amino acid replacement, insertion, or deletion within a

range where desired N-methyl transferase activity is not damaged in the amino acid sequence of SEQ ID NO: 1 can be mentioned. That is, polypeptides having an amino acid sequence of SEQ ID NO: 1 having the desired N-methyl transferase activity as described above and those having a modified sequence are referred to as N-methyl transferase.

The above polypeptide having the modified amino acid sequence itself has functions substantially identical to N-methyl transferase of cotyledons of tea, and has high homology with an amino acid sequence of SEQ ID NO: 1 at a site associated with enzyme activities.

In general, between a plurality of enzymes having the identical functions, it is well known that homology of the amino acid sequence other than a site indispensable for enzyme activities is very low (Kawagoe et al., Proc. Natl. Acad. Sci. USA, 93, 12082- (1996)). Therefore, even in the case where the entire homology is low, transferase having high homology at a site associated with activities can be classified as N-methyl transferase.

In comparing the entire amino acid sequence, there can be exemplified a modified amino acid sequence which can provide a polypeptide having the desired N-methyl transferase and homology of 15% or more, preferably homology of 30% or more, more preferably homology of 45% or more, and further preferably homology of 60% or more, and still further preferably homology of 75% or more, the most preferably homology of 95% or more against the amino acid sequence of SEQ ID NO: 1.

In the case where modification against the amino acid sequence of SEQ ID NO: 1 as the basis are defined at a level of nucleotide sequence encoding the modified amino acid sequence, there can be provided a modified nucleotide sequence having homology of 40% or more, preferably homology of 60% or more, more preferably homology of 75% or more, further preferably homology of 90% or more, still further preferably homology of 95% or more against the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1.

A nucleotide sequence encoding the N-methyl transferase according to the present invention, that is, an N-methyl transferase gene can contain a nucleotide

sequence encoding one of the an amino acid sequences of SEQ ID NO: 1. A specific example thereof can include DNA sequence of SEQ ID NO: 2 and RNA sequence of SEQ ID NO: 3. A nucleotide sequence having homology [ruled] as described above [against] with the N-methyl transferase genes as the basis is also included [in] with the gene encoding the N-methyl transferase according to the present invention.

As a modified amino acid sequence maintaining the desired N-methyl transferase activities, its preferable examples include that encoded by a modified nucleotide sequence, which can hybridize under stringent conditions with the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 as the basis.

In addition, as the modified N-methyl transferase gene, that capable of hybridizing with the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 under stringent conditions can be preferably utilized in practice. A specific example thereof can include DNA molecules capable of being hybridized under stringent conditions [for] to a nucleotide sequence of SEQ ID NO: 2; and RNA molecules capable of being hybridized under stringent conditions [for] to a nucleotide sequence of SEQ ID NO: 3.

Hybridization under these stringent conditions can be carried out, for example, by the method described in Molecular Cloning: Cold Spring Harbor Laboratory Press, Current Protocols in Molecular Biology; Wiley Interscience. As a commercially available system, a GeneImage system (Amasham) can be exemplified. Specifically, hybridization can be carried out by the following operation.

A membrane to which DNA or RNA molecules to be tested has been transferred is treated for hybridization using a labeled probe in a hybridization buffer specified by the protocol in accordance with product protocols. The composition of hybridization buffer consists of 0.1 wt. % SDS; 5 wt.% Dextran sulfate; 1/20 volume of a blocking reagent included in the kit and 2 to 7 x SSC. A blocking reagent is used by preparing 100 x Denhardt's solution, 2% (weight/volume) Bovine serum albumin, 2% (weight/volume) Ficll™ 400, 2%

(weight/volume) polyvinyl pyrrolidone at a 5-fold concentration, and diluting them to 1/20. 20 x SSC is a 3M sodium chloride and 0.3M citric acid solution. SSC is preferably used at a concentration of 3 to 6 x SSC, and further preferably used at a concentration of 4 to 5 x SSC.

The hybridization temperature ranges from 40 to 80°C, more preferably 50 to 70°C, and further preferably 55 to 65°C. Incubation for several hours or one night is carried out, followed by washing using a washing buffer. The washing temperature is preferably equal to room temperature, and more preferably is a temperature during hybridization. The composition of thee washing buffer is a 6 x SSC + 0.1 wt.% SDS solution, more preferably 4 x SSC + 0.1 wt. % SDS solution, further preferably 2 x SSC + 0.1 wt. % SDS solution, still further preferably 1 x SSC + 0.1 wt.% SDS solution, and the most preferably 0.1 x SSC + 0. 1 wt. % SDS solution. A membrane is washed by such washing buffer, whereby the DNA molecules or RNA molecules in which the probe is hybridized can be identified by utilizing a label employed for the probe.

Modification may occur in nature or may be artificially generated by site mutation in the nucleotide sequence.

DNA molecules having N-methyl transferase genes according to the present invention can be separated from cells producing N-methyl transferase according to the present invention by utilizing PCR technique as described in "Plant PCR test protocols" (another volume of cell engineering, plant cell engineering series 2) Shujynsha (1995), in which an oligo nucleotide specifically hybridized for DNA molecules for encoding N-methyl transferase, for example, is employed as a primer.

Specifically, a linker is bonded with cDNA synthesized from mRNA, and PCR is carried out between the linker and the DNA encoding an amino acid sequence constituting the N-methyl transferase, whereby the full length sequence of the target cDNA can be isolated.

DNA molecules encoding the N-methyl transferase obtained by such hybridization technique or PCR technique have homology with the N-methyl transferase gene of SEQ ID NO: 2 at least at a site used for isolation. The

homology used herein denotes homology of 15% or more, preferably homology of 30% or more, more preferably homology of 45% or more, further preferably homology of 60% or more, still further preferably homology of 75% or more, still furthermore homology of 90% or more, and the most preferably homology of 95% or more in comparing the amino acid sequence encoded by the respective N-methyl transferase genes. However, even if homology with N-methyl transferase becomes 15% or less as a result of deletion, addition, and replacement of a plurality of residues of amino acid to be encoded, it is estimated that some of the obtained N-methyl transferase genes maintain a region indispensable to the functions of N-methyl transferase, and substantially encode proteins having their functions similar to N-methyl transferase.

All of the organisms employed for isolating DNA molecules or RNA molecules having nucleotide sequences (genes) encoding N-methyl transferase according to the present invention can be used as long as they produce caffeine or its precursor. They can include Theaceae Camellia plant such as tea, Rubiaceae Coffea plant such as coffee, Sterculiaceae Cola plant such as Cola or the like.

A RNA molecule including a N-methyl transferase gene according to the present invention can be obtained by connecting a DNA encoding the N-methyl transferase, which can be prepared by the above described method, in the desired direction at the operable location on the downstream side of a promoter such as Sp6 promoter or T7 promoter, which is recognized by RNA polymerase, to prepare a recombinant molecule and translating the recombinant molecule by Sp6 RNA polymerase or T7 polymerase to obtain the desired RNA molecule. The RNA molecule can be also obtained by introducing a DNA or RNA encoding the N-methyl transferase into a plant virus or inserting a DNA or RNA encoding the N-methyl transferase into a vector carrying an appropriate expression cassette as described below so that the DNA or RNA is connected to the expression cassette in the desired direction at the operable location to prepare a recombinant molecule and introducing the recombinant molecule into a host microorganism or plant so that the RNA encoding the N-methyl transferase can be formed in the host using the transcription activity of the host.

DNA molecules having complementarity with all or a part of the DNA molecules having the N-methyl transferase genes or RNA molecules having complementarity with all or a part of the RNA molecules having N-methyl transferase genes can be used as DNA or RNA molecules for inhibition or suppression of N-methyl transferase expression in plant cells as long as these molecules have a function to inhibit expression of the N-methyl transferase peculiar to the host plant cells when these molecules are expressed in the host plant cells.

According to the present invention, the part of the N-methyl transferase gene as the basis for the complementary DNA or RNA molecule denotes a site which can be used to provide a complementary sequence as a basis to form mRNA for inhibition (i.e., antisense RNA) in the host cells. This mRNA for inhibition is formed in host cells, the RNA is bonded with mRNA for expressing N-methyl transferase in host cells, and the expression of N-methyl transferase in the host cells is inhibited. This site is necessary for forming such antisense mRNA, which has, for example, at least 14 base length.

DNA molecules for forming the [antisence] <u>antisense</u> mRNA can include, for example, DNA molecules complementary to all or a part of the nucleotide sequence SEQ ID NO: 2, and [antisence] <u>antisense</u> RNA molecules include RNA molecules complementary to all or a part of the nucleotide sequences of SEQ ID NO: 3. DNA molecules or RNA molecules having complementarity with all or a part of the modified sequence capable of being hybridized with these nucleotide [sequence] sequences of SEQ ID NOs: 2 and 3 under stringent conditions can also be used for such purpose.

The DNA molecules having high homology with these inhibiting DNA molecules or RNA molecules and having desired inhibiting or restricting functions can also be utilized. Here, high homology denotes homology of 60% or more, preferably homology of 75% or more, further preferably homology of 90% or more, and the most preferably homology of 95% or more in comparing the respective nucleotide sequences.

These inhibiting DNA molecules or RNA molecules themselves may not always encode N-methyl transferase according to the present invention.

Another aspect of the nucleotide sequence for inhibiting N-methyl transferase according to the present invention is directed to a nucleotide sequence having one or more sites with homology to the N-methyl transferase gene, but do not encode N-methyl transferase. The N-methyl transferase peculiar to the host cells can be inhibited by replacing this nucleotide sequence with the N-methyl transferase gene of the host cells to be deleted.

With respect to expression of the N-methyl transferase gene or expression of the DNA molecules having functions for inhibiting or restricting expression of the N-methyl transferase gene or N-methyl transferase in the host cells, an example utilizing plant cells as host cells will be described hereinafter.

For expression in plant cells, there can be utilized a method for introducing into host plant cells an expression cassette and transforming the host cells, which expression cassette includes: (i) a promoter enabling transcription from DNA to mRNA in the host cell; (ii) a DNA fragment containing a N-methyl transferase gene bonded with the downstream side of the promoter in the sense or [antisence] antisense direction or a DNA fragment having functions for inhibiting the expression of N-methyl transferase; and (iii) a terminator sequence containing a [polyadenilation] polyadenylation site required for stabilization of transcribed products bonded with the downstream side of these DNA fragments as required.

Such expression cassette and a vector containing this cassette are the subject of the present invention.

The expression cassette can contain a promoter for constitutively or inductively expressing the inserted DNA. In addition, this expression cassette can have a replication origin for its replication in the plant cells as required.

Promoters for constitutive expression include, for example, a 35S promoter for cauliflower mosaic virus, rice actin promoter and the like. In addition, promoters for inductive expression include, for example, promoters known to be expressed by external factors such as infection or invasion of mold, bacteria or virus, low temperature, high temperature, dry, anaerobic conditions, specific compound spraying or the like. Such promoters include a promoter of chitinase genes of rice expressed, for example, by infection or invasion of mold, bacteria or

virus; a promoter of PR protein genes; a promoter of "lipl9" genes of rice induced by low temperature; a promoter of "HSP182" genes of Capsella bursa-pastoris induced by high temperature; a promoter of "rab" genes of rice induced by dry; a promoter of alcohol dehydrogenase gene of corn induced by anaerobic conditions or the like. In addition, the promoter for the chitinase genes of rice and the promoter for PR protein genes of tobacco are induced by specific compounds such as salicylic acid or the like, and the rice "rab" gene promoter is induced by spraying abscisic acid of plant hormone.

Alternatively, as a promoter for expressing DNA inserted into the expression cassette, a promoter derived from any of the N-methyl transferase genes per se can be used.

A specific example of promoter isolation can include a method f or selecting genome DNA fragments and specifying the DNA at the upward section of the gene by utilizing the hybridization technique in which all or a part of N-methyl transferase genes is employed as a probe.

In order to prepare for introduction of recombinant DNA molecules in the expression cassette into plants, there can be utilized a number of cloning vectors containing a replication signal in E. coli and a marker gene(s) for selection of the transformed E. coli cells. Examples of such vectors include pBR322, pUC system, Mi3mp system or the like. A target sequence can be introduced at a proper restriction enzyme cutting site. In order to clarify characteristics of the obtained plasmid DNA, analysis of the restriction enzyme cutting site, gel electrophoresis, and other biochemical, molecule-biological methods are generally used. After each operation has been completed, the plasmid DNA is cut, and can be bonded with another DNA. The sequence of each plasmid DNA can be cloned in the same plasmid or another plasmid.

In order to introduce the expression cassette into plant cells, a variety of techniques can be used. These techniques include transformation of plant cells with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes as transformation factors; direct induction into protoplast (injection method,

electroporation method or the like), Particle gun method or the like and any other possibility.

In direct introduction into protoplast, no vector is particularly required. For example, a simple plasmid such as pUC derivative can be employed. Some methods for introducing target genes into plant cells may require another DNA sequence. For example, when Ti or Ri plasmid is employed for transformation of plant cells, it is preferable to connect at least right-end sequence of the T-DNA region for the Ti and Ri plasmids, mostly the sequences on both ends so as to be adjacent regions of genes to be introduced.

When Agrobacterium is employed for transformation, an expression cassette to be introduced is required to be cloned in a specific plasmid, that is, in an intermediate vector or a binary vector. The intermediate vector is not replicated in the Agrobacterium. The intermediate vector is moved into the Agrobacterium by a helper plasmid or an electroporation. The intermediate vector has a region homologous with the sequence of T-DNA, and thus, is incorporated into the Ti or Ri plasmid of Agrobacterium by homologous recombination. Agrobacterium used as a host is required to include a vir region. In general, the Ti or Ri plasmid includes the vir region, and T-DNA can be moved to plant cells by way of the functions of the region.

On the other hand, the binary vector can be replicated and maintained in Agrobactrilium. Thus, when the binary vector is incorporated into Agrobacterium by the helper plasmid or electroporation technique, T-DNA on the binary vector can be moved to plant cells by the functions of the Vir region of the host.

The intermediate vector or binary vector including the thus obtained expression cassette and microorganisms such as Escherichia coli or Agrobacterium including these vectors are the subject of the present invention.

The transformed plant cells can be converted into a plant tissue or, a plant body by experiencing a reproduction process. The reproduction methods depend on the kinds of plant cells, and include the Fujimura et al. method for rice (Plant Tissue Culture Lett., 2. 74- (1995)); the Shillito et al. method for corn

(Bio/Technology, 7, 581- (1989)) ; and the Akama et al. method for Capsella bursa-pastoris (Plant Cell Rep., 12, 7- (1992)) or the like.

According to the present invention, the term "plant body" means the whole individual organism classified into plant or organ parts thereof such as leaves, stems, roots, flowers, fruits, seeds and the likes.

For the plant body produced by these methods or the plant body obtained from its breeding catalyst (for example, seeds, stems, cutting or the like), an expression quantity of N-methyl transferase according to the present invention changes in comparison with a wild type plant body producing caffeine or its precursor; a change in generation quantity of caffeine metabolism system compounds due to modification of metabolism of the host plant or a change in production rate of the caffeine metabolism system compound group due to modification of metabolism of the host plant takes place. The thus-obtained transgenic plant is the subject of the present invention. The plants according to the present invention includes specific tissues or cells of plants such as leaves, flowers, fruits, seeds or the like.

In addition, in recent years, from study on post-translation gene silencing of plants, it has been found that the expression of target genes can be restricted by utilizing the intrinsic protection mechanisms of the plant for exotic nucleic acids such as virus (Cell, 95, 177-187 (1998), Chemistry and Biology, 37, 532- (1999) and protein nucleic acid enzyme, 44, 1396- (1999). According to this study, in the case where DNA virus or RNA virus or the like invades plants, the plants transcribe aberrant RNA from these molds, and double-strand RNA is formed sequentially specifically with the transcribed product of the intrinsic sequence that plants possess. This double-strand RNA is decomposed by RNase, thereby making it possible to restrict the expression of target genes (Cell, 96, 303 - (1999)). One of the essential characteristics of this method is that a sequence whose expression is to be restricted does not always need to be transformed to the target plant. In addition, further characteristics of this method is that, if a target nucleic acid is introduced into part of the plant (low-order leaf or the like) by infection or the like, its effect widens the entire plant body. A specific expression restricting method is

to cause double-strand RNA including all or part of the sequence of the target gene or the sequence having high homology or Agrobacterium having the double-strand DNA to be infected with the low- order leaf of the plant. Here, high homology denotes homology of 60% or more, preferably homology of 75% or more, further preferably homology of 90% or more, and the most preferably homology of 95% or more in comparing the respective base sequences.

In the plant body subjected to this method, an expression quantity of the N-methyl transferase protein according to the present invention changes in comparison with a wild type plant body producing caffeine or its precursor. In addition, change in expression quantity of the caffeine metabolism compounds due to modification of metabolism of the host plant or change in production rate of caffeine metabolism system compound group due to modification of metabolism of the host plant takes place. The thus-obtained plant is the subject of the present invention. The plants used in this invention include specific tissues or cells of plants such as leaves, flowers, fruits, seeds or the like.

Plants producing caffeine with the SAM being a methyl group donor can include caffeine [productive] producing plants including Theacea Camellia plants such as tea; Rubiaceae coffea plants such as coffee; Sterculiaceae Cola plants such as Cola.

Microorganisms for introducing DNA that encodes the N-methyl transferase, thereby expressing a large amount of the N-methyl transferase proteins according to the present invention can include bacteria such as E. coli, Bacillus subtilis or the like; and virus such as Baculoviridae.

In addition, any plants producing caffeine or its precursor can be used as plants into which a DNA that encodes the N-methyl transferase according to the present invention in the sense or antisense form to obtain transformed plants for the purposes of improving productivity for a specific compound and changing a production ratio of a specific compound group by modifying the metabolism in host cells.

The production of secondary metabolites relating to the caffeine synthesis system and the composition of the secondary metabolites produced by these

transformants can be modified by culturing plant cells or plant tissues transformed with the vector of the above described constitution or cultivating plants transformed in the same manner. As the secondary metabolites, for example, at least one compound selected from the group consisting of 7-methyl xanthine, paraxanthine, theobromine, and caffeine can be mentioned.

As supply sources of plant cells, plant tissues, or plant bodies used for transformation, for example, a Camellia plant, a Coffea plant, Cola plant, Ilex plant, Neea plant, Firmiana plant, Paulinia plant, or Therbroma plant body can be mentioned as plant bodies for transformation.

These plants can include Theaceae Camellia plants such as tea; Rubiaceae coffea plants such as coffee; Sterculiaceae Cola plants such as Cola or the like.

Further, in the N-methyl transferase according to the present invention, structurally analogous compounds such as 7-methyl xanthine as well as theobromine can be methylated. Thus, even if they are not the above kinds of plants, the method according to the present invention is applicable to plants containing structurally analogous compounds of xanthine thereof.

As a result of enzymological study, it is clear that the N-methyl transferase according to the present invention has the following basic properties.

Molecular weight: 41,000 (SDS-PAGE), 61,000 (gel filtration)

Isoelectric point: 4.5 to 5.0 (Chromatofocusing)

Optimum pH: 8.5

Km value: 21 μM (SAM) or 24 μM (paraxanthine)

Inhibitor: SAH (S-adenosyl homocysteine)

Reaction mechanism: SAM + Paraxanthine → SAH caffeine

Examples

Hereinafter, the present invention will be described in more detail by way of Examples and Comparative Examples. The scope of the present invention is not limited to these Examples.

Example 1

Preparation of Purified Fraction of N-Methyl Transferase

First, second, and third leaves of tea leaves (Camellia sinensis var. Yabukita) collected in Makurazaki City, Kagoshima Prefecture, Japan, in May, 1997 were frozen using liquid nitrogen, and preserved at -80°C. This material of 100 g was milled by adding 5 mM EDTA-Na_2 of 1,000 ml; 2.5 mM 2-mercapto-ethanol; 5% (v/v) glycerin; 1 mg aprotinin; 0.5% (w/v) sodium ascorbate; and 50 mM sodium phosphate buffer solution (pH 7.3) containing 2.5% (w/v) insoluble polyvinyl polypyrrolidone, and was filtrated through three-layer gaze. Then, the filtered liquid was centrifuged (10,000 g, 15 minutes), and a supernatant was obtained. Then, a 50-80% saturated ammonium sulfate fraction was prepared from the supernatant. This fraction was deionized using Sephadex G - 25; was dissolved in 10 mM sodium phosphate buffer solution (pH 7.2) containing 2 mM EDTA-Na_2; 2 mM 2-mercapto-ethanol; 20% (v/v) glycerin; and then, was adsorbed to hydroxy apatite column (15 x 160 mm) equilibrated with the same buffer solution; and an active fraction was eluted using a 10-200 mM linear concentration gradient of 200 ml of the sodium phosphate buffer solution containing 2 mM EDTA-Na_2; 2mM 2-mercaptoethanol; 20% (v/v) glycerin. The active fraction was collected, [precipitation] <u>precipitate</u> was recollected by 80% unsaturated ammonium sulfate, and the fraction was dissolved in 50 mM Tris - hydrochloric acid buffer solution (pH 8.4) containing 2 mM EDTA-Na_2; 2 mM 2-mercapto-ethanol; 20 mM KC1; 20% (v/v) glycerin. After deionization was carried out, and the fraction was adsorbed to a cathode ion exchange column Shodex IEC QA-824 (8 x 25 mm) for the exclusive use for high performance liquid chromatography balanced by the same buffer solution. After the column was washed by the same buffer solution, the adsorbed protein was eluted by a linear concentration gradient 30 ml of 20 - 750 mM KC1 (dissolved in the 50 mM Tris - hydrochloric acid buffer solution (pH 8.5) containing 2 mM EDTA-Na_2; 2 mM 2-mercapto-ethanol; and 20% (v/v) glycerin). The active fraction was collected, deionized, and adsorbed by adenosine-agarose (1 ml) that is an affinity column balanced by the same buffer solution after being dissolved in 50 mM Tris - hydrochloric acid buffer solution (pH 8. 5) containing 2 mM EDTA-Na_2; 2 mM 2-mercapto-ethanol; and 20% (v/v) glycerin. The active fraction was eluted by the 50 mM Tris - hydrochloric acid buffer solution (pH 8.5)

containing 0.2 M NaCl; 2 mM EDTA-Na_2; 2 mM 2-mercapto-ethanol; and 20% (v/v) glycerin. The obtained fraction was subjected to gel filtration using HiLoad Superdex 200 (16 x 600 mm) balanced by the 50 mM Tris - hydrochloric acid buffer solution (pH 8.5) containing 2 mM 2-mercapto-ethanol; 150 mM KC1; 20% (v/v) glycerin, and a finally refined sample was obtained. Table 1 summarizes a change in rate activity in the refining process of N-methyl transferase.

Steps	Fraction	Liquid quantity (ml)	Total activity (pkat)	Total protein mass (mg)	Specific activity (pKat/mg)	Degree of Purification	Production rate (%)
1	Crude Extract	930	6330	581	10.9	1.0	100
2	Ammonium sulfate	33.8	3410	155	22.0	2.0	53.9
3	Hydroxy apatite	23.0	2630	28.9	91.0	8.0	41.5
4	Shodex IEC QA-824	7.5	1070	4.82	221	20.3	16.9
5	Adenosine - agarose	2.0	202	0.08	2530	232	3.2
6	Superdex 200	5.8	228	0.04	5700	523	3.6

Example 2

Analysis of Amino Acid Sequence of N-Methyl Transferase Refined Fraction

The finally-purified sample was transferred to a PVDF membrane using a Semidry Blotting apparatus after SDS-polyacrylamide gel electrophoresis. A site at which N-methyl transferase was transferred was cut away, and an amino acid sequence of an N-terminal was analyzed using an ABI protein sequencer. The result is shown in SEQ ID NO:4 (Phe Met Asn Arg Gly Glu Xaa Glu Ser Ser Tyr Ala Gln Asn Ser Gln Phe Thr Gln Val).

Example 3

19 residue oligo nucleotide NMT-1 and Not I- (dT) 18 primer (Pharmacia Biotech) based o 7-amino acid residue of the N-terminal of the oligo nulceotide for cDNA cloning of N-methyl transferase was employed as a probe. The sequence of NMT-1 is shown in SEQ ID NO:5.

Example 4

Synthesis of single strand cDNA for cloning of N-methyl transferase

(1) Isolation of total RNA

Young tea leaves of 5 g were milled under the existence of liquid nitrogen using rod or mortar. After sublimation of the liquid nitrogen, 3M LiCl of 50 ml and 8 M urea were added, and was further milled by polytron. After the milled leaves were statically placed one night at 4°C, and were centrifuged at 12,000 rpm for 15 minutes. [Precipitation] Precipitate was suspended in a 0.5% SDS, 10 mM Tris - hydrochloric buffer solution (pH 7.6) to be about 10 ml in total amount. The suspended [precipitation] precipitate was mixed by adding 10 ml phenol/chloroform solution, and was centrifuged at 12,000 rpm for 10 minutes. A 1/10 volume of 3M sodium acetate solution (pH 4.8) and then 2 volumes of ethanol were added to the supernatant, the mixture was statically placed at -80°C for one hour. Centrifugation was carried out at 4°C and 12,000 rpm for 10 minutes, 70% ethanol was added to the [precipitation] precipitate separated from the supernatant to form suspension, and centrifugation was carried out again. The supernatant was

removed, and the residue was dried up by a vacuum pump. The [precipitation] precipitate thus obtained was dissolved in 1.5 ml water, 150 μl of 3M sodium acetate solution (pH 4.8) was added thereto, and further, 1.5 ml of phenol/chloroform solution was added thereto to be fallen and mixed. Then, centrifugation was carried out at 12,000 rpm for 10 minutes. Ethanol of x2 volume was added to the supernatant, and was statically placed at -80°C for 20 minutes. Then, centrifugation was carried out at 40°C and 12,000 rpm for 10 minutes. To the thus obtained [precipitation] precipitate, 70% ethanol was added, and centrifugation was carried out again. The [precipitation] precipitate was dried up by te vacuum pump, was dissolved in 200μl water, and thus, a fraction of total RNA was obtained.

(2) Isolation of MRNA

The total RNA (2 mg) obtained by the above described method was subjected to heat treatment at 65°C for 5 minutes, and then, was mixed with solution A of the same volume of 2-fold concentration (10 mM Tris - hydrochloric acid buffer solution (pH 7.5); 1 mM Na_2 - EDTA; 0.1% SDS; 0.5 M NaCl). 0.1 g of oligo (dT) - Cellulose Type 7 (Pharmacia) was immersed in 2 ml of solution B (10 mM Tris - hydrochloric acid buffer solution (pH 7.5); 1 mM - EDTA-Na_2; 0.1% SDS; 0.1 M NaCl), and the suspension was poured into a blue chip filled with glass wool at its tip end, and was washed by 2.5 ml of 0.1N NaOH. Then, 5 ml of solution A was poured and balanced. Total RNA was applied to this column, and 3 ml of solution A and 4 ml of solution B were poured. Then, mRNA was eluted by 3 ml of solution C (10 mM Tris - hydrochloric buffer solution (pH 7.5), 1 mM - EDTA-Na_2, and 0.05% SDS). The eluted solution was condensed by ethanol precipitation and was dried up. Then, the solution was dissolved in water, and was preserved at -80°C.

(3) Synthesis of Single-Strand cDNA

190 ng of MRNA was acutely cooled for 3 minutes immediately after heat treatment at 65°C for 10 minutes. This sample was employed as a template, and a single-strand cDNA was synthesized using First-Strand cDNA Synthesis Kit (Pharmacia). The synthesized cDNA was preserved at -20°C.

Example 5

Cloning of N-Methyl Transferase Genes Using the RT-PCR Technique

The following reaction solution containing the single strand cDNA prepared by the above described method as a template was prepared. Oligonucleotide NMT-51 having 19 residues (5'-ttYatgaaYMgIggIgIgaRg-3') based on the N-terminal 7 amino acid residues of the purified N-methyl transferase in Example 2 and NotI-(dt)18 primer (Pharmacia Biotech) were used as the primers. This reaction solution was reacted at 95°C for 1 minute using Peltier Thermal cycler PTC-200 (Funakoshi) and then PCR was carried out under conditions in which one cycle included the steps of 95°C/1 minute, 45°C/1 minute, and 72°C/2 minutes, respectively and reacted by 30 cycles, and the reacted product was obtained.

Template cDNA: 3 μl

10 x buffer: 5 μl

2.5 mM NTP: 8 μl

NMT-1: 1 μl (50 pmol)

NotI- (dT) 18: 1 μl (50 pmol)

H_20: 31 μl

ExTaq (TAKARA): 1 μl

Example 6

Sub-cloning to Plasmid Vector

Electrophoresis of the reaction product obtained in Example 5 was carried out in TAE using 0. 8% agarose gel, a band of the obtained target product was cut away, and DNA was recollected from a gel using GENE CLEAN (Funakoshi). The recollected DNA was transformed to Escherichia coli DH5α after ligation with pT7blue vector (Novagen). After color selection was carried out using X-gal, liquid culture was carried out in the LB culture medium containing Ampicillin, and plasmid was extracted using the alkali-SDS technique. The presence or absence of the insert was verified by agarose electrophoresis.

Example 7

Nucleotide Sequence Determination

Primer extension was carried out in the following reaction solution using the isolated plasmid. The reaction conditions are as follows. After reaction at 96°C/l 5minute, PCR in which one cycle included the steps of 96°C/0. 2 minute, 50°C/0.1 minute, and 60°C/4 minutes, respectively, and repeated by 25 cycles was carried out. Ethanol precipitation was carried out for the reaction solution, and the obtained DNA was dissolved in Template suppression reagent, and was analyzed using ABI-310 genetic analyzer. In order to determine a sequence at the center of 10the target DNA, there was used a plasmid in which the DNA fragment obtained by treating DNA with Styl was sub-cloned in pUC19. These primer sequences are shown in SEQ ID Nos: 6 and 7.

Composition of the Primer Extension Reaction Solution

Plasmid DNA (20 ng): 2 μl

15 Premix: 4 μl

Primer: 1 μl

H_20: 3 μl

Example 8

Isolation of 5' Upstream Area of N-Methyl Transferase mRNA Using 5' the 20RACE Technique

5'- Full RACE Core Set (TAKARA) was employed for isolation of the 5' upstream area. The sequences of the primers used for SEQ ID NOS: 8 to 17 [was shown] are shown below.

With the lst strand cDNA synthesized by the method described in Example 254, after decomposition of hybrid RNA and cycling of the single strand cDNA with ligation reaction, PCR reaction was carried out based on normal technique using the primers of SEQ ID Nos: 8 to 12 or SEQ ID NOs: 13-17, and the reacted product was obtained. A band of the reacted product was separated by acryl amide electrophoresis, and DNA was recollected f rom a gel, and was sub-cloned to the

pT7blue vector. Then, the nucleotide sequence of the DNA inserted by the technique similar to those in Examples 6 and 7 was determined.

The DNA sequence of the N-methyl transferase gene thus obtained from tea and the amino acid sequence encoded by the gene are shown as SEQ ID NO: 18.

Example 9

Expression of N-Methyl Transferase in Escherichia coli

The following operation was carried out in order to re-incorporate the isolated N-methyl transferase gene into the expression vector pET23d (Novagen).

PCR was carried out under the following conditions using the pT7blue vector into which the isolated N-methyl transferase gene DNA obtained in Example 6 as a template and the primers of SEQ ID NOs: 18 and 19, respectively, and the reaction product [were] <u>was</u> obtained.

Reaction conditions:

After reaction at 95°C/1.5 minutes, PCR in which one cycle included the steps of 95°C/1 minute, 52°C/1 minute, and 72°C/1 minutes, respectively, and repeated by 30 cycles were carried out.

Separately, a fragment obtained by treating the isolated N-methyl transferase gene DNA fragment with NcoI and EcoRI was inserted into pET23d vector. Then, the above PCR product was [further] inserted into the NcoI site of this pET23d vector to construct a N-methyl transferase expression plasmid. This plasmid was transformed to Escherichia coli BL21 (DE3). After the obtained Escherichia coli was cultured at 37°C for 2 hours, IPTG was added thereto so as to be 0.3 mM in final concentration, and 3-hour culture was further carried out at 30°C. After culture [has] <u>had</u> been completed, cells were collected, and the cells from 3 ml culture solution was subjected to supersonic treatment for 1 minute with interval in 0.2 ml 10 mM Tris - hydrochloric acid buffer solution (pH 7.5), 0.1 M NaCl, 1 mM EDTA-Na_2. Then, centrifugation was carried out at 14,000 rpm for 10 minutes, and the obtained supernatant was employed as an enzyme solution.

Regarding the sequencing in Examples 7 and 8, the nucleotide sequence of the DNA fragment including the N-methyl transferase gene obtained in this example

has the sequence of SEQ ID NO:2 and the corresponding RNA nucleotide sequence has the sequence of SEQ ID NO:3. The corresponding amino acid sequence of the N-methyl transferase is shown as SEQ ID NO:1.

The reaction solution for N-methyl transferase measurement was prepared as follows. 10 μl of enzyme solution was added to 100 mM Tris - hydrochloric acid buffer solution (pH 8.5), 0.2 mM $MgCl_2$, 0.2 mM paraxanthine, 4 μM [methyl-^{14}C] S - adenosyl methionine (0. 9 kBq) , and the volume of the reaction solution was 100 μl. Reaction was carried out at 27°C for 10 minutes, the obtained ^{14}C - caffeine was extracted by adding 1 ml chloroform thereto, and the radiation activity of a chloroform layer was measured. As a control, xanthosine was added to the reaction solution instead of paraxanthine or paraxanthine was removed. As a result of measurement of activity, it was found that 1.56 pmol of caffeine was produced only when paraxanthine was added as a substrate.

The above enzymatic reaction was repeated except that 7-methyl xanthine or theobromine was added to the reaction solution instead of paraxanthine. As a result of measurement of enzyme activity, production of theobromine was observed in the reaction solution to which 7-methyl xanthine was added instead of paraxanthine, while production of caffeine was observed in the reaction solution to which theobromine was added instead of paraxanthine. Regarding these results, it was revealed that the enzyme isolated in the above procedure had three different N-methyl transferase activities using two other substrates in addition to paraxanthine, respectively.

Example 10

Suppression of Caffeine Synthesis According to an Antisense Method

A recombinant vector carrying an antisense N-methyl transferase gene was constructed by the following procedure:

DNA fragments were amplified by PCR using the total length of the isolated N-methyl transferase gene as used in Example 9 as the template and the primer having the nucleotide sequences of ID SEQ NOs: 20 and 21, respectively. The

ends of the DNA fragments thus amplified were changed into the blunt ends by BKL kit (TAKARA) to obtained the blunt -ended PCR amplified fragments.

Separately, pBI vector (Clontech), to which a hygromycin resistance gene connected, was cut with XbaI and SacI to remove the β-glucuronidase gene and the 5ends of the linear vector thus obtained was changed into the blunt ends.

The blunt-ended linear vector was ligated with the blunt-ended PCR amplified fragments to obtain recombinant vectors by Ligation Kit (TAKARA), and then the vector carrying the desired N-methyl transferase gene which was inserted in the reverse direction at the operable location on the downstream side of the 10CaMV35S promoter in the pBI vector was selected from the reaction products by sequencing. Thus, the desired recombinant vector, into which the antisense N-methyl transferase gene was inserted, was obtained and used the following transformation:

Regarding the conventional methods of biological synthesis of caffeine using 15coffee tissue culture, there have been many reports such as Planta, 108, 339 (1972), Plant Cell Reports, 2, 109 (1983). Coffee callus was induced from a coffee shoot apex or a young leaf according to the conventional methods. The recombinant vector was introduced into the callus thus obtained by the particle gun method. Alternatively, protoplasts of the callus was prepared by the conventional methods 20and the recombinant vector was introduced into the protoplasts by electroporation. After the introduction, cells having the make resistance were selected. The cells thus selected were cultured under light condition and the enzyme activity of the transformed cells was measured according to the method as described in Example 9. As a result, it was revealed that the caffeine production by the transformed cells 25into which the antisense N-methyl transferase gene was introduced was significantly reduced in comparison with that of the normal cells into which the antisense N-methyl transferase gene was not introduced.

Redifferentiation of the transformed coffee cells were conducted to obtain a young coffee plant, whereby the redifferentiation was carried out by a conventional 30methods described in the reports including Z. Pflanzenphysiol. Bd., 81, 395 (1977); and Plant Cell, Tissue and Organ Culture, 8, 243 (1987). The activity of

each of the enzymes from the leaves of he young coffee plant was measured. As a result, it was revealed that caffeine production of the young coffee plant, into which the antisense N-methyl transferase was introduced, was significantly reduced in comparison with that of the young coffee plant, into which the antisense N-methyl 5transferase gene was not introduced.